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Effect of *Astragalus polysaccharide* and its sulfated derivative on growth performance and immune condition of lipopolysaccharide-treated broilers

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ABSTRACT

This study evaluates the immunomodulating activities of *Astragalus polysaccharide* (APS) and sulfated APS (SAPS) in LPS-infected broiler chicks. SAPS was derived using the classic chlorosulfonic acid–pyridine method. On day 16, the birds were injected intramuscularly with 0.5 mL of either saline, APS (4 or 8 mg/kg of body weight (BW), shorten as APS-4 or APS-8) or SAPS (4 or 8 mg/kg of BW, shorten as SAPS-4 or SAPS-8) once a day for three successive days. On days 19 and 20, the birds were intraperitoneally injected with 0.5 mL of LPS (1 mg/kg of BW). Saline was used as blank control. Compared with the blank control, LPS-treated birds showed lower daily body weight gain (BWG), average daily feed intake (ADFI), villus height and intraepithelial lymphocytes (IEL) number in jejunum, and higher feed conversion ratio (FCR, feed:gain), spleen index, plasma NO concentration, blood heterophil:lymphocyte (H:L) ratio, and the production of NO in the blood T lymphocytes. Compared with the LPS group, birds in APS-4, SAPS-4 and SAPS-8 groups showed decreased FCR ($P < 0.05$). Moreover, SAPS increased BWG and jejunal villus height ($P < 0.05$) at 8 mg/kg BW. Plasma NO concentration was lower in APS-8 group than that in LPS group ($P < 0.05$). Both APS-8 and SAPS-8 treatments elevated the number of jejunal IEL ($P < 0.05$), and decreased blood H:L ratio ($P < 0.05$), respectively. Administration of APS or SAPS did not affect the ADFI, immune organ index, crypt depth and mucosal thickness of the jejunum, and the number of goblet cell. Our findings suggested that APS and SAPS possessed dose-dependent growth-promoting and immunomodulating effect, and was a potential development direction for immunomodulator under early LPS stimulation condition.

1. Introduction

Enteric diseases are great threat to the poultry industry, resulting in altered immune function, lower productivity, higher mortality, and the contamination of poultry products. Gram-negative bacteria LPS is the leading cause of enteric diseases. LPS can initiate a cascade of intracellular signal transduction after binding to the extracellular domain of TLR4, and promote the secretion of enormous cytokines, such as TNF-α, IL-1β and IFN-γ [1], resulting in immune stress. Monocytes [2], macrophages [3], neutrophils [4] and endothelial cells [5] were also activated by LPS, which activated innate immune system.

As a common plant in many regions of China, *Astragalus membranaceus* has been used as a traditional tonic to enhance the body's innate immune functions. *Astragalus polysaccharide* (APS) is one of the main active ingredients of *Astragalus membranaceus*, and has been licensed in clinic application in China for many years due to its extensive range of biological activities, such as anti-inflammatory [6], anti-bacterial [7], anti-oxidant [8], anti-parasitic [9], anti-carcinogenic [10], and especially immunomodulatory activities [11]. Birds fed with 220 mg/kg APS showed improved humoral and cellular immunity [12]. APS possesses immune modulating activity by promoting spleen lymphocytes proliferation in rats with gastric cancer [13]. Zhang et al. [14] found that mice pre-administration with APS showed increased DC maturation, T lymphocytes proliferation, and the phagocytic capacity of peritoneal macrophage, indicating enhanced immune status. APS modulates the innate immunity through TLR4 signal pathway in urinary tract under mucosal bacterial infection [15].

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Polysaccharides are large-molecular-weight polymers with specific spatial structures. The bioactivity of the polysaccharide is directly affected by its structure, such as structural units, glycosidic bond, molecular weight, and conformation. Moderate structure modification could enhance the antitumor, antioxidant, antiherpetic and adjuvant activities in carboxymethylated [16], acetylated [17], phosphorylated [18] and sulfated [19] polysaccharide derivatives, respectively. Sulfated modification is derived by adding a sulfated group on its original hydroxyl group through classic chlorosulfonic acid–pyridine method. Sulfated polysaccharides are proven to possess enhanced bioactivities, such as immunopotentiator [19], anti-oxidant [17], anti-coagulant [20], antitumor [16], and anti-HIV [21].

In our previous study, we obtained sulfated APS (SAPS) by chlorosulfonic acid–pyridine method, and proven its enhanced bioactivity in LPS-infected Caco2 cells *in vitro* [22]. However, the immunomodulatory effect of SAPS is still not elucidated, especially *in vivo*. So, in the present study, we use 180 LPS-challenged Arbor Acres broiler chicks as an *in vivo* model to explore the immunomodulating activities of APS and SAPS.

2. Materials and methods

2.1. Reagents and instruments

APS with the purity of 97%, molecular weight of 20,000–60,000 Da, and endotoxin content less than 0.1 EU/mg was bought from Pharmagenesis Inc., USA. *Escherichia coli* 055:B5 LPS (L2880) and ConA (C5275) were derived from Sigma–Aldrich, St. Louis, MO. Wright–Giemsa and lymphocyte separation medium were the products of Beijing Solarbio Science & Technology Co., Ltd, Beijing, China and Tian Jin Hao Yang Biological Manufacture Co., Ltd, Tianjin, China, respectively. Commercial kit for measurement of nitric oxide (NO) was from Jiancheng Bioengineering Institute, Nanjing, China.

Leica microtome was derived from Leica Instruments GmbH, Hubloch, Germany. Nikon 80i microscope and NIS-Elements Documentation software were the products of Nikon, Tokyo, Japan.

All other reagents were of analytical grade.

2.2. Preparation of SAPS

APS modification was carried out using the chlorosulfonic acid–pyridine method according to our previous research [22]. Briefly, the sulfated reagent (chlorosulfonic acid/pyridine, v/v, 1/6) was prepared in an ice bath. Then, 400 mg APS was added to the sulfated reagent after mixed with N,N-dimethylformamide. The mixture was stirred for 1 h at 95 °C, terminated with ice-cold distilled water, neutralized with saturated NaOH, and precipitated with absolute ethyl alcohol. The solution was dialyzed in dialysis sack against tap water and distilled water for 48 h and 12 h, respectively, and lyophilized to obtain SAPS.

2.3. Structural analysis of SAPS

2.3.1. Degree of substitution (DS) determination

First, the content of sulfur (S%) was determined using colorimetric method according to Dodgson [23], taking sodium sulfate as standard. Then, the DS of SAPS was calculated according to the following equation: $DS = (1.62 \times S\%)/(32 - 1.02 \times S\%)$. The DS of SAPS was 1.4 in the present research.

2.3.2. Fourier transform infrared spectroscopy (FT-IR) spectrum analysis

The spectrum analysis was conducted using the KBr pellet method in AVATAR 330 FT-IR Thermo Nicolet instruments (Thermo Electron Corporation, Madison, WI) in the range of 400–4000 cm^{-1}. The two characteristic absorption bands of SAPS at 1265 and 810 cm^{-1} represented the asymmetrical S=O stretching vibration, and symmetrical C—O—S vibration, respectively, indicating that APS was successfully sulfated (Figs. 1 and 2).

2.4. Birds and experimental design

This study was approved by the Animal Care and Use Committee of Northwest A&F University. In total, 180 one-day-old healthy Arbor Acres broiler chicks were purchased from Shaanxi Zhengda Co., Ltd, China, and randomly assigned to 6 treatments (6 replicates/treatment, 5 birds/replicate). All birds were allowed *ad libitum* access to a commercial diet and 24 h access to water. The diet was formulated to meet or exceed the NRC (1994) recommendations and was devoid of antibiotics. Bird management was consistent with recommendations of Arbor Acres Broiler Commercial Management Guide. For the first 7 days, the environment temperature in the chicken house was set at 33 °C, and reduced by 3 °C each week until 24 °C.

The research was composed of two stages, nutrient treatment stage and LPS challenge stage. Stage one was conducted from 16 to 18 days, the birds in 5 experimental groups were intramuscularly injected 0.5 mL of saline (LPS group), APS (4 or 8 mg/kg of body weight (BW), expressed as APS-4, or APS-8) or SAPS (4 or 8 mg/kg of BW, expressed as SAPS-4, or SAPS-8), respectively. The control group was injected in 0.5 mL of saline. All birds were injected one time a day for three days. Stage two was conducted from 19 to 20 days, the birds in 5 experimental groups were intramuscularly injected with 0.5 mL of LPS (1 mg/kg of BW) to induce an immune stress. The control group was injected 0.5 mL of saline. All birds were injected once a day for two successive days.

2.5. Sampling procedure

Daily body weight gain (BWG), average daily feed intake (ADFI) and feed conversion ratio (FCR, feed:gain, g:g) from 16 to 21 days were calculated. Chick mortality was recorded daily. On day 21, one bird from each replicate was randomly selected for blood sampling. For each bird, about 10 mL blood was collected from the wing vein and transferred immediately to a heparinized centrifuge tube. Blood heterophil:lymphocyte (H:L) ratio was calculated using Wright–Giemsa staining method according to Zhang et al. [24]. In brief, 5 μL blood was used to make a smear on a glass slide. The smear was stained with Wright–Giemsa. A total of 100 heterophils and lymphocytes were counted under a microscope at a magnification of 100× with oil immersion by a trained person. The H:L ratio was then calculated.

About 8 mL blood was centrifuged at 3000 × *g* for 10 min to get plasma. The concentration of plasma NO was measured using a commercial kit according to the manufacture's instruction.

About 1 mL blood from each sample was used to separate lymphocytes using lymphocyte separation medium. The medium was centrifuged at 2000 × *g* for 25 min after diluted with equal volume of Hanks' solution. The cells were washed, resuspended and seeded to the concentration of 1×10^7/mL with 6 wells for each sample. Then, 20 μL LPS (50 μg/mL) or ConA (25 μg/mL) was added to stimulate the proliferation of B lymphocytes or T lymphocytes, respectively. The supernate was collected 48 h later for the examination of NO concentration using a commercial kit according to the manufacture's instruction.

The birds were then killed by cervical dislocation; the thymus, spleen and bursa were removed and weighed immediately